Using GFP in FRET-based applications

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The use of green fluorescent protein (GFP) is a powerful technology that has recently enabled investigators to study dynamic molecular events within living cells. One method for detecting molecular interactions involves fluorescence resonance energy transfer (FRET) between two GFPs or between GFP and a second fluorophore. This review summarizes the use of GFP for FRET and illustrates the theme with specific examples on how GFP has been employed as an intracellular molecular sensor.

Green fluorescent protein (GFP) has served as a versatile tool in cell biology for studying gene expression, as well as protein folding and trafficking (reviewed in Ref. 1). The utility of GFP in research applications derives from its intrinsic ability to generate fluorescence in live tissues, in the absence of any cofactor(s). By far the most frequent applications of GFP have involved its use as a genetically encoded tag, where GFP is fused in-frame to a protein of interest, and the resulting chimeric protein is expressed in an appropriate cellular background for examining protein function and fate. Fortunately, GFP can be targeted to most subcellular sites, making it feasible to examine biochemical processes in real time.

In addition to these passive applications, GFP has also been used as an active indicator of cellular physiology. This might seem surprising given its extraordinarily rigid tin-can-like structure[2,3], which would be predicted to be relatively insensitive to environmental signals. Wild-type GFP protein has not been particularly useful as a sensor, but several GFP mutants with distinct spectral qualities have been used as environmental sensors in various situations – for example, in monitoring local pH[4]. The most powerful method of constructing GFP sensors has been to exploit fluorescence resonance energy transfer (FRET) between two GFP molecules or between one GFP molecule and a secondary fluorophore. FRET is a phenomenon that occurs when two fluorophores are in sufficient proximity (<100 Å) and an appropriate relative orientation such that an excited fluorophore (donor) can transfer its energy to a second, longer-wavelength fluorophore (acceptor) in a nonradiative manner. Thus, excitation of the donor can produce light emission from the acceptor, with attendant loss of emission from the donor. Because FRET is a nondestructive spectroscopic method for measuring molecular interactions, it can be done in living cells, as represented by the ratio between donor and acceptor emission intensities.

GFP mutants used as FRET pairs

Five basic classes of useful GFP mutants have been described[1]. Briefly, they are:

- GFP (designated herein as eGFP), which can have different substitutions of the Ser65 residue (e.g. Thr, Ala, Gly). The emission peak is most like that of wild-type GFP around 511 nm but lacks the near-UV 395 nm excitation peak;
- B(lue)FP, a group of blue-shifted GFPs with a Y66H mutation in the chromophore;
- C(yan)FP, a group of GFPs carrying a Y66W mutation, with excitation and emission spectra intermediate to those of BFP and eGFP;
- Sapphire, a mutant with the 495 nm excitation maximum suppressed, leaving only the short-wavelength (395 nm) excitation peak. This mutant still emits at a GFP-like wavelength (511 nm);
- Y(ellow)FP, a group of GFP mutants carrying an aromatic amino acid at position 203 and with red-shifted spectra.

The excitation and emission spectra of these GFP families with relative absorption or fluorescence brightness, respectively, are shown in Fig. 1.

When selecting GFPs to use as workable FRET pairs, three spectroscopic properties of the donor and acceptor GFPs should be considered. First, there needs to be sufficient separation in excitation spectra if the donor GFP is to be stimulated selectively. Second, there needs to be an overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor to obtain efficient energy transfer. Third, reasonable separation in emission spectra between donor and acceptor GFPs is required to allow the fluorescence of each chromophore to be measured independently. These guidelines are not absolute since attainment of all of these qualities between two GFPs is never perfect. Rather, it is the combination of these properties that is important, which can permit reproducible monitoring of GFP-based FRET by ratiometric measurement.

To date, two GFP pairs have been used in FRET-based biological systems. BFP is often employed as the donor, and eGFP as the acceptor. Although this GFP pair has experienced the broadest use (due in part to their availability from commercial suppliers), BFP is only weakly fluorescent. This is a serious limitation for its use in applications outside of those employing microscopy or flow cytometry for fluorescence detection. An alternative GFP–FRET pair that has been used successfully in a variety of situations is the CFP–YFP combination[1]. CFP is significantly brighter than BFP, permitting accurate ratiometric measurement of both donor and acceptor GFPs by a variety of fluorescence detectors, including plate-type readers. However, a limitation of the CFP–YFP FRET system is the extenuated tail for the right end of the CFP emission spectrum (Fig. 1). Bleeding of the CFP emission into the emission spectrum of YFP compromises the independent

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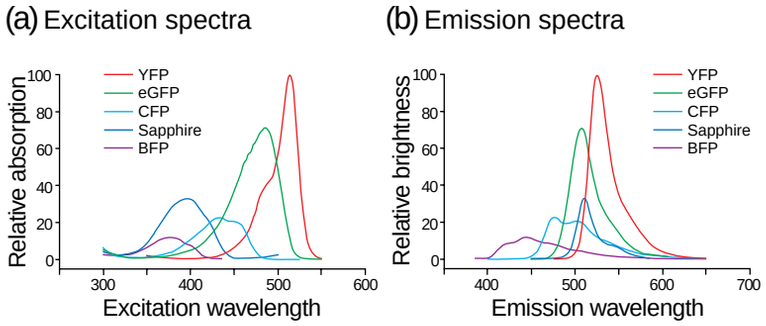

FIGURE 1

Relative excitation (absorption) and emission (brightness) spectra for the five green fluorescent protein (GFP) mutant classes. Purified recombinant protein was used to generate the spectra. Spectra are represented in the following colours: BFP, violet; Sapphire, royal blue; CFP, cyan; eGFP, green; YFP, red. Y-axis values are set relative to YFP (100%). (Reproduced, with permission, from Ref. 14.)

measurement of fluorescence derived from the donor and acceptor GFPs, thus decreasing the efficiency of detecting FRET changes. Judicious choice of filter sets for fluorescence detection helps to minimize this problem.

Tandem GFP FRET pairs used as protease substrates

The earliest use of GFP as a FRET donor and/or acceptor was in the genetic construction of FRET-based endoprotease substrates. Heim and Tsien[5,6] demonstrated that a BFP (Y145F, Y66H) donor and an eGFP (S65C or S65T) acceptor could engage in FRET when they were linked by a 25-amino-acid sequence. Complete cleavage within this linker sequence by trypsin destroyed FRET, leading to a 4.6-fold change in the ratio of fluorescence emission from the respective GFPs (535 nm for eGFP, 440 nm for BFP). Mitra *et al.*[7] used a similar GFP–FRET pair to construct a tandem BFP–eGFP substrate for Factor Xa. They observed a threefold change in the 505 nm : 450 nm emission ratio.

Because GFP can be expressed in a variety of mammalian cell types, both authors postulated that tandem GFP molecules connected by a cleavable linker sequence for a particular protease could serve potentially as intracellular reporters for cytoplasmic- or Golgi-localized proteases[5,7]. Recent work by Xu *et al.*[8] has demonstrated the feasibility of this approach. They showed that FRET was lost in apoptotic cells transiently expressing a tandem BFP–eGFP caspase-3 substrate linked together by a peptide sequence that included the DEVD recognition site for caspase-3. The absence of ratiometric data in this paper makes it is difficult to assess the efficiency of this intracellular protease reporter relative to earlier tandem GFP protease substrates and also illustrates the inherent difficulty in measuring BFP fluorescence within cells.

A comparative *in vitro* protease digestion experiment using CFP–YFP and BFP–YFP tandem molecules is shown in Fig. 2. Spectroscopic data before and after cleavage illustrate how the bleeding of the CFP can compromise the measured FRET change due to protease cleavage of the tandem GFP substrate (compare the near-complete decrease in acceptor fluorescence at 530 nm in the BFP–YFP pair with the much smaller 530 nm fluorescence change for the CFP–YFP pair). Even though the overall emission ratio change after complete cleavage is better for the BFP–YFP pair (5-fold vs 4.2-fold for CFP–YFP), the improved brightness and photostability of the CFP donor makes the CFP–YFP pair the superior protease reporter for use in cells.

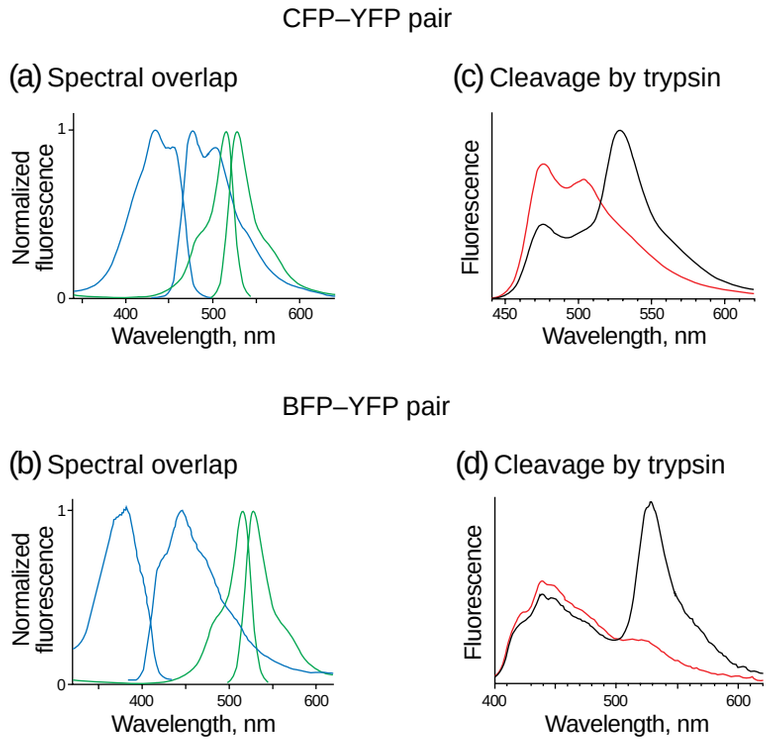

FIGURE 2

Spectra for two tandem green fluorescent protein (GFP) protease reporters, CFP–YFP and BFP–YFP. (a, b) Donor (blue lines) and acceptor (green lines) excitation and emission spectra. These spectra illustrate how the CFP–YFP pair has better overlap for donor emission and acceptor excitation, but poorer separation of donor and acceptor emission spectra, than the BFP–YFP pair. (c, d) Overall emission spectra of each GFP–FRET pair before (black) and after (red) cleavage with trypsin. The more efficient energy transfer in the CFP–YFP pair leads to a larger observed change in the donor CFP emission (425/500 nm peaks). The better emission separation in the BFP–YFP pair, however, gives a greater decrease in the acceptor YFP emission (530 nm peak). A fourfold ratio change for each of the tandem GFP protease substrates is seen with trypsin, showing that FRET readout with two GFPs is a balancing act between several biophysical characteristics of the proteins. These analyses were performed on a SPEX FluoroLog-2 instrument.

Tandem GFP pairs used as sensors of protein–protein interactions

Day used BFP and eGFP fusion proteins to measure static homodimerization of the Pit-1 transcription factor in HeLa cells[9]. Although detection of homodimerization is inherently more difficult to attain than heterodimerization – at most 50% of complexes will involve BFP–eGFP interaction – careful microscopy and analysis detected BFP–eGFP FRET.

Another system for examining protein–protein interactions was developed independently by Romoser *et al.*[10] and by Miyawaki *et al.*[11] in which a GFP FRET pair reported changes in intracellular Ca^{2+} concentration. Romoser linked BFP to eGFP with a 26-residue spacer containing the calmodulin-binding domain from smooth muscle myosin light chain kinase (MLCK). A basal level of FRET occurred within this tandem GFP fusion protein, probably because the flexible linker allowed the two GFP molecules to dimerize. Addition of Ca^{2+}–calmodulin to this reporter protein disrupted FRET, as evidenced by a sixfold change in the emission ratio. This is dramatic given that the GFPs remain physically connected after binding of the Ca^{2+}–calmodulin protein. Protein binding could alter the orientation rather than the separation distance of the BFP donor and eGFP acceptor and thus significantly affect FRET efficiency[1]. This biochemical system was reconstituted in HEK/293 cells by microinjection of purified recombinant protein. The ratio change was much more modest in cells, but the experiment showed that BFP–eGFP FRET can be measured in living cells.

In the second case, Miyawaki *et al.* fused either BFP or CFP to the N-terminus of calmodulin, and eGFP or YFP to the C-terminus of M13, the calmodulin-binding peptide of MLCK. This system of genetically encoded Ca^{2+} sensors was dubbed 'cameleons' by the authors. Increased intracellular Ca^{2+} concentration caused binding of the M13 peptide to calmodulin, which increased FRET. After demonstrating the performance of the system in a biochemical mode, they expressed the cameleon proteins in transiently transfected HeLa cells; using GFPs optimized for brightness and mammalian expression, the total fluorescence from these proteins produced a signal sufficient to allow use of DNA-mediated transfection rather than microinjection of protein. The CFP+calmodulin–YFP+M13 peptide pair was clearly superior in detecting Ca^{2+}-induced intracellular FRET. The maximal emission ratio change was 1.3-fold when the two GFPs were linked, as compared with a ratio change of nearly twofold upon association of two separate GFP fusion proteins. When GFP association occurs *in trans*, the local concentration of the GFP fusion proteins influences FRET change, with expression of equimolar amounts of each GFP fusion protein being ideal. FRET imaging of individual cells stably expressing the linked version of yellow cameleon protein is shown in Fig. 3.

Because GFP fusions can be expressed *in situ*, they can be targeted to subcellular organelles such as the endoplasmic reticulum (ER), where there are Ca^{2+} stores. By adjusting the Ca^{2+} affinities through mutation of the calmodulin protein and establishing that the emission ratio correlated with Ca^{2+} concentration, the authors measured the free [Ca^{2+}] in both the cytoplasm and ER. They found that the free [Ca^{2+}] in the ER was 60–400 μM in unstimulated cells, falling to 1–50 μM in cells treated with Ca^{2+} ionophores (e.g. ionomycin).

Recently, protein interactions within mitochondria have been visualized by GFP–FRET. Mahajan *et al.*[12] constructed fusion proteins of eGFP–Bax and BFP–Bcl-2 to demonstrate directly that Bax and Bcl-2

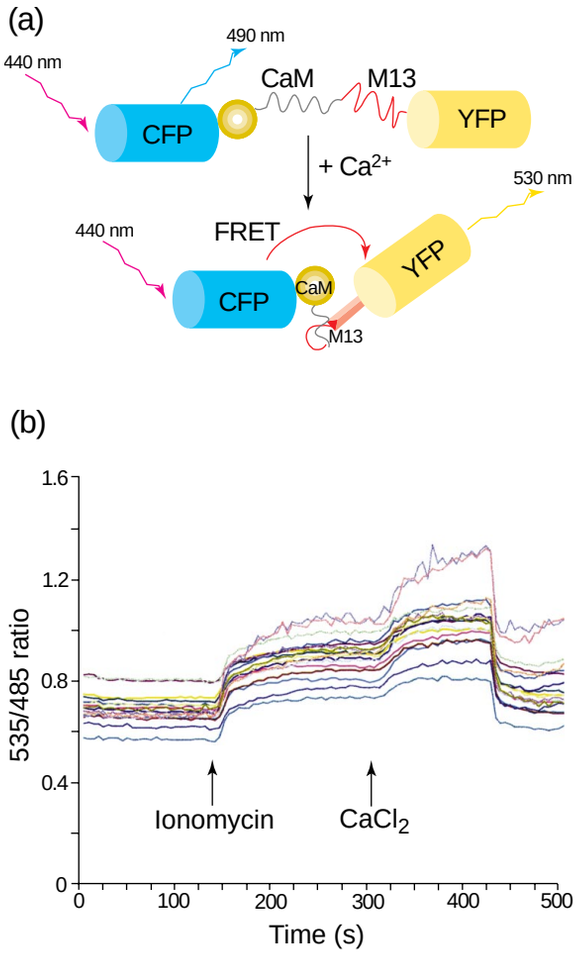

FIGURE 3

(a) Schematic depiction of cameleon system for measuring protein–protein interactions by fluorescence resonance energy transfer (FRET). Increasing local [Ca^{2+}] leads to association of the intervening calmodulin protein and the M13 myosin light chain kinase peptide, with accompanying increase in FRET between CFP and YFP. (b) FRET response of individual HEK/293 stable transfectant cells, expressing yellow cameleon-3 protein[11], to addition of a Ca^{2+} ionophore (1 μM ionomycin) and additional extracellular Ca^{2+} (30 mM). Heterogeneity in the Ca^{2+}-driven FRET response within this pool of stable transfectants can be overcome by isolation of clones by flow cytometry. Single-cell clones expressing high levels of yellow cameleon-3 protein ($\geq 5 \times 10^5$ molecules $cell^{-1}$) provides a more homogeneous and responsive (1.4-fold ratio change) cell population (J. Jones, unpublished). Imaging was performed on a Zeiss Axiovert 135 TV microscope.

proteins can associate in living cells close enough for FRET to occur. Because Bcl-2 and Bax GFP fusion proteins appear to possess wild-type biological activities, this approach should allow study of the temporal aspects of Bax–Bcl-2 protein association during apoptosis.

GFP in FRET applications with other fluorophores

Some mutants of GFP are well suited for FRET with conventional fluorescent dyes such as fluorescein or tetramethylrhodamine. Until recently, however, use of these dyes has required purification of the protein to be labelled. The recent work by Griffin, Adams and Tsien[13] in generating a system for specific labelling of recombinant proteins *in situ* has opened up the feasibility of using GFP in intracellular FRET-based experiments with a fluorescein-like protein label. The label, designated FLASH–EDT_2, is cell-permeable and covalently labels recombinant proteins containing an artificial, 17-amino-acid tetracysteine-based peptide tag. The FLASH fluorophore has excitation and emission maxima similar to those of fluorescein, making CFP a potential FRET donor. Griffin *et al.* demonstrated that cytoplasmic-localized CFP protein containing a C-terminal FLASH-peptide tag could be labelled efficiently by FLASH–EDT_2 and that FRET was measured readily from the 480 nm CFP : 635 nm FLASH emission ratio. Additional advantages of the FLASH reagent are its nonfluorescent nature in the unbound form, the ability to control protein labelling quantitatively *in situ* and the flexibility to substitute other fluorophores in place of fluorescein.

From FLASH to enlightening the future

Considerable progress has been made using GFP–FRET as a means of generating genetically encoded sensors within living cells. Future progress in this area will probably come through use of improved GFP mutants as well as new types of fluorescent proteins. Commercial application of GFP sensors has been slower to develop owing to the significant challenge of using GFP in cell-based assays where detection is not based on microscopy. Many investigators have had difficulty obtaining sufficient signal above plate background and cellular autofluorescence when using conventional 96-well plate readers. Judicious deployment of flow cytometry to isolate high-expressing clones can obviate this problem, but also advances in detection imagers for high-throughput screening should provide an independent solution. Nevertheless, basic research applications using GFP–FRET should continue to grow thanks to the exciting work validating GFP as a real-time reporter of biochemical events within living cells.

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